# Electroacupuncture improves synaptic plasticity by regulating the 5-HT1A receptor in hippocampus of rats with chronic unpredictable mild stress

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## Abstract

**Objectives:** To investigate the antidepressant effects of electroacupuncture (EA) on chronic unpredictable mild stress (CUMS) in rats, as well as the effects of EA on hippocampal neurons, synaptic morphology, and 5-hydroxytryptamine (HT) receptor expression.

**Methods:** Forty adult male Wistar rats were randomly divided into normal control, CUMS, EA, and paroxetine groups. CUMS modeling was performed for 21 days, followed by 14 days of intervention: rats in the EA group underwent stimulation of GV20 and GV29 acupuncture points for 30 minutes daily; rats in the paroxetine group were administered paroxetine daily. Behavioral tests, transmission electron microscopy, western blotting, and real-time quantitative polymerase chain reaction were used to evaluate the effects of the intervention.

**Results:** EA treatment reversed the behavioral changes observed in rats due to CUMS modeling; it also improved the pathological changes in organelles and synaptic structures of hippocampal

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neurons, and upregulated the protein and mRNA expression levels of 5-HT1A receptor. There were no significant differences in 5-HT1B receptor protein and mRNA expression levels among the groups.

**Conclusions:** EA treatment can alleviate depression-like symptoms in CUMS rats. The underlying mechanism may include promoting the expression of 5-HT1A receptor mRNA and protein, thereby improving synaptic plasticity in the hippocampus.

## Introduction

Depression is a common mental disorder with combined point, 1-year period, and lifetime prevalences of 12.9%, 7.2%, and 10.8%, respectively; it is associated with a high economic burden.[1,2] Work-related disability and loss of productivity due to depression contribute substantially to the economic costs of depression.[3] Antidepressants are the main therapeutic agents for depression;[4,5] however, a major barrier to antidepressant treatment is that approximately one-third of affected patients discontinue medication because of ineffectiveness or adverse reactions.[6] Furthermore, rapid antidepressants cause safety and ethical concerns.[7,8] Thus, more effective and safer antidepressant treatment strategies are needed.

There is increasing evidence that the pathogenesis of depression is closely related to hippocampal synaptic plasticity.[9] One study revealed reduced hippocampal volumes in depressed patients, which were associated with neuronal atrophy, synaptic dysfunction, and synaptic loss.[10] Serotonin is an important neuroregulatory transmitter that strongly influences synaptic plasticity; 5-hydroxytryptamine (HT) can reverse these changes by interacting with the corresponding receptor to regulate synaptic plasticity.[11,12] Extracellular 5-HT levels can increase rapidly,[13] leading to the activation and response of postsynaptic and presynaptic 5-HT receptors (i.e., 5-HT1A and 5-HT1B receptors) in many brain regions.[14,15] Recent research has also shown that novel antidepressants modulate brain-derived neurotrophic factor levels in the hippocampus and induce neurogenesis in this region.[16,17] Therefore, we presume that the hippocampus is an important brain region for the treatment of depression.

Acupuncture is a characteristic traditional Chinese medicine therapy that has been proven effective in the treatment of depression.[18–21] Electroacupuncture (EA) has been shown to enhance stress-induced reductions in hippocampal 5-HT levels in animal models.[22] Previously, we found that electricity has an antidepressant-like effect in depressed patients or animals; moreover, the antidepressant mechanism of EA is related to hippocampal synaptic plasticity.[23,24] The 5-HT receptor contributes to serotonin-mediated regulation of synaptic plasticity, as well as modulation of 5-HT levels.

To further confirm the efficacy of EA as an antidepressant and to explore possible mechanisms underlying the therapeutic effects of EA, we analyzed the effects of EA on hippocampal synaptic plasticity and the expression levels of 5-HT1A and 5-HT1B receptors. Here, we used the

chronic unpredictable mild stress (CUMS) model of depression, which is effective, reliable, and widely used.[25] The CUMS model resembles the human experience of depression and has been validated in previous antidepressant studies.[26,27] Governor vessel (GV) 20 and GV 29 were selected for EA intervention, because these acupoints are commonly used for treatment of depression.[28]

## Materials and methods

### Animals

Forty 6-week-old male Wistar rats (weight $200 \pm 20$ g, from the Experimental Animal Center of Southern Medical University) were used in this experiment. All rats were housed in single cages under a 12-hour/12-hour light/dark cycle in a standard specific pathogen-free facility (temperature, $24 \pm 2°C$; humidity, 50% to 60%); good indoor ventilation was ensured during feeding times. Rats were subjected to adaptive feeding for 7 days before the experiment. At the end of the adaptive feeding period, rats with greater behavioral differences were identified with the aid of behavioral indicators; these rats were excluded from the experiment. All animal procedures were conducted in accordance with the National Institutes of Health Guide for Animal Care and Use, and were approved by the Ethics Committee on Animal Experimentation of Southern Medical University (approval no. L2015056).

### Groups

Rats were randomly divided into four groups: normal control (n = 10), CUMS (n = 10), EA (n = 10), and paroxetine (n = 10). Rats in the normal control group were raised normally without any treatment; they received food and water ad libitum throughout the experiment. The remaining groups were subjected to 21 days of CUMS exposure (i.e., the modeling period). A 14-day intervention of EA or paroxetine was conducted after completion of CUMS modeling (Table 1).

### CUMS procedure

With the exception of rats in the normal control group, all rats were randomly exposed to 10 different stressors within 21 days.[29,30] The schedule of randomly selected stressors is shown in Table 2. Following stimulation, CUMS rats were evaluated by means of a behavioral assessment (described in the subsection "Behavioral assessment").

### Interventions

Rats in the EA group were treated with EA at GV 20 (above the apex auriculate, on the midline of the head) and GV 29 (at the middle point between the eyes) beginning on day 22 (1 day after CUMS modeling was complete), once per day for 30 minutes (Figure 1).[19,22] Acupuncture needles (0.30 mm × 25 mm, Hwato Brand, Suzhou Medical Instrument Factory,

**Table 1.** Schedule for animal experiment.

|  | Normal | CUMS | EA | Paroxetine |
|---|---|---|---|---|
| Days 1–21 | Normal feeding | CUMS modeling | | |
| Day 22 | | Behavioral tests | | |
| Days 22–35 | No intervention | | EA intervention | Paroxetine intervention |
| Day 36 | | Behavioral tests, tissue collection | | |

Suzhou, China) pierced the skin horizontally at GV 20 and GV 29 at a depth of 5 mm to Deqi. During needling, electrodes were connected to the needle handle; the Econtinuous wave current had a frequency of 2 Hz and intensity of 0.6 mA for 30 minutes per treatment. Rats in the paroxetine gro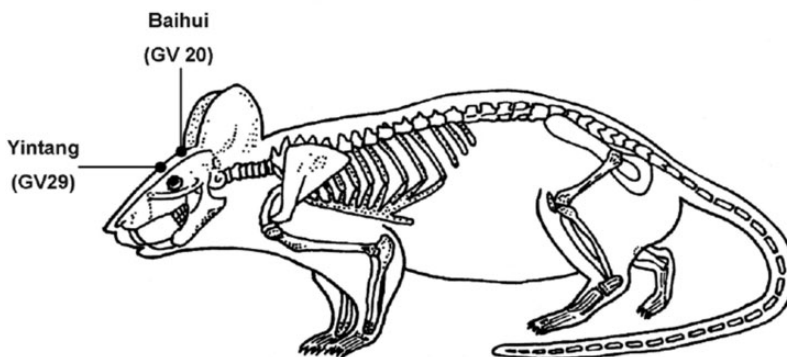up underwent daily intragastric administration of paroxetine (1.8 mg/kg/day, SK & F, Tianjin, China), following 30 minutes of immobilization to ensure treatment similar to that of the EA group.[31] The CUMS group was also immobilized for 30 minutes, as in the EA group. All interventions were performed once per day for 14 days (from day 22 to day 35).

### Behavioral assessment

Behavioral tests were conducted twice (i.e., after modeling and after intervention) to evaluate the effects of these experimental manipulations. Before the sucrose preference test, rats were trained to drink in a quiet environment from two identical water bottles in each cage. For the first 24 hours, both bottles contained the same amount of 1% sucrose solution (200 g). For the second 24 hours, one bottle contained 1% sucrose solution (200 g) and the other one contained water (200 g). The rats were then deprived of water and food for 23 hours. The test phase began on the following morning: rats were administered the same amounts of 1% sucrose solution

**Table 2.** Schedule for CUMS modeling procedure.

| Stressor | Day | | |
|---|---|---|---|
| Water deprivation for 24 hours | 10 | 21 | |
| Fasting for 24 hours | 1 | 20 | |
| Wet padding for 24 hours | 6 | 13 | |
| Inversion of the light/dark cycle for 24 hours | 2 | 16 | |
| Stroboscopic illumination for 12 hours | 7 | 15 | |
| Physical restraint for 4 hours | 3 | 11 | 17 |
| Swimming in cold water at 4°C for 5 minutes | 5 | 14 | |
| Swimming in warm water at 45°C for 5 minutes | 9 | 18 | |
| Level shaking for 5 minutes | 4 | 12 | |
| Tail nip for 3 minutes | 8 | 19 | |



**Figure 1.** Diagram of rat acupoints.
This diagram was drawn in accordance with the schematic published in Experimental Acupuncture Science (Shanghai Scientific & Technical Publishers).
Yu SG and Guo Y. Experimental Acupuncture Science. 2nd ed. Shanghai, China: Shanghai Scientific & Technical Publishers, 2014, p146.

(200 g) and water (200 g), and the liquid consumption (in grams) was measured after 1 hour. Sucrose preference was calculated by the ratio of sucrose solution consumed to total liquid consumed.

In the open field test, the open field test box was placed in a quiet and dark room to avoid the influence of sound and light during the experiment. The field was divided equally into 25 grids, and each grid was numbered from left to right. The central region consisted of nine grids and the peripheral region consisted of 16 grids (central region:peripheral region = 9:16). Each rat was placed gently in the center of the field to observe the time spent in the central region and total activity for 5 minutes. The entire recording and evaluation process was completed using a video camera (SSG-G218, Sony, Beijing, China) and related software (Smart 3.0, Panlab, Cornellà, Spain).

## Transmission electronic microscopy (TEM)

TEM was used to observe the morphological structure of hippocampal tissue after CUMS modeling and subsequent interventions. Rats were anaesthetized by intraperitoneal injection of 2% pentobarbital sodium (0.3 mL/100 g). Then, rats received aortic infusions of 0.9% normal saline and cooled 4% polyformaldehyde. Hippocampal tissues were collected and fixed in 2.5% glutaraldehyde.

To prepare specimens for TEM, hippocampal tissues were rinsed in 0.1 mol/L phosphoric acid and fixed in 1% osmium tetroxide. Subsequently, tissues were dehydrated using ascending concentrations of acetone and ethanol, then embedded in epoxy resin. Embedded hippocampal tissues were cut in semi-thin sections (0.5–2 μm), then stained with uranyl acetate and lead citrate. Sections were photographed by a transmission electron microscope (H-7500, Hitachi, Tokyo, Japan).

## Western blotting analysis

Protein levels of 5-HT1A and 5-HT1B receptors were measured by western blotting. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium (0.3 mL/100 g), then decapitated. Hippocampal tissue was immediately removed and frozen in liquid nitrogen. Frozen hippocampal tissue was thawed and lysed in lysis buffer (Beyotime Biotechnology, Shanghai, China); lysates were centrifuged at 14,000 ×g for 10 minutes at 4°C, and supernatants were collected for subsequent detection. Protein concentrations were measured using the Pierce® BCA Protein Assay Kit (KeyGene, Nanjing, China). Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 10× Tris-buffered saline and 20% Tween-20, diluted in distilled water (TBST) for 1 hour at room temperature; they were then incubated with primary antibody overnight at 4°C (anti-5-HT1A, Cat. No. GTX104703, 1:1500 dilution; anti-5-HT1B, Cat. No. GTX100009, 1:1000 dilution; GeneTex, Irvine, CA, USA). Subsequently, membranes were washed three times with TBST (10 minutes per wash) and then incubated with secondary antibody, goat anti-rabbit IgG (horseradish peroxidase-conjugated, 1:20,000 dilution; SouthernBiotech, Birmingham, AL, USA), for 1 hour at room temperature. Membranes were again washed three times with TBST (10 minutes per wash); protein bands were then visualized by enhanced chemiluminescence with Immobilon Western HRP Substrate (Cat. No. WBKLS0500; Millipore), using X-ray film. Band intensities were quantified by optical densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

## Quantitative real-time polymerase chain reaction (PCR) analysis of mRNAs

Quantitative real-time PCR was used to evaluate mRNA expression levels of 5-HT1A and 5-HT1B receptors. Methods of anesthesia and tissue collection in rats were the same as those described in western blotting analysis. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions.[32] Eppendorf tubes were loaded with 5 μL total RNA, 1 μL primer, 1 μL of 1 nM dNTP Mix (Life Technologies, Carlsbad, CA, USA), and 5 μL diethyl pyrocarbonate-treated water. To achieve reverse transcription, the tubes were incubated in a 65°C water bath for 5 minutes, then incubated for 1 minute at 0°C. Subsequently, 4 μL of 5x first-strand buffer, 2 μL of 0.1 M dithiothreitol, 1 μL RNaseOUT, and 200 μL SuperScript III RT (all purchased from Life Technologies) were added to each tube; the tubes were incubated at 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 15 minutes in quick succession, followed by immediate freezing at 0°C.

Quantitative real-time PCR was performed after reverse transcription, using the primer sequences shown in Table 3; the Real-time Quantitative PCR Detection System (Life Technologies) was used to perform amplification and detection. β-actin was used as the reference gene to normalize the 5-HT1A and 5-HT1B data.

Amplification conditions were initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 59°C for 30 seconds. After amplification, agarose gel electrophoresis was performed to verify that amplification had occurred; the bands were analyzed under ultraviolet light. SYBR Green real-time PCR Master Mix (Life Technologies) was used for quantitative real-time PCR to evaluate the concentrations of PCR amplification products. Results were calculated using the $2^{-\Delta\Delta CT}$ method and β-actin was used to normalize the values. The normal control group was used for calibration, such that the relative expression levels of 5-HT1A and 5-HT1B receptor in the control group were defined as 1; the expression levels in other groups were calculated as fold changes relative to the levels in the normal control group.

## Statistical methods

Data were analyzed and processed using SPSS Statistics, version 20.0 (IBM Corp., Armonk, NY, USA); the results are expressed as mean ± standard deviation. All data were evaluated for statistically significant differences using one-way analysis of variance followed by least significant difference post hoc tests, or Dunnett's post hoc test if heteroscedasticity was present (as determined by the F-test). Differences with $P < 0.05$ were considered statistically significant.

**Table 3.** Sequences of primers for quantitative real-time polymerase chain reaction.

| Gene | Primer name | Primer sequence |
|------|-------------|-----------------|
| 5-HT1A | 1.5-HT1A-F | TCCGACGTGACCTTCAGCTA |
| | 1.5-HT1A-R | GCCAAGGAGCCGATGAGATA |
| 5-HT1B | 2.5-HT1B-F | CCGGCTAACTACCTGATCGC |
| | 2.5-HT1B-R | TATCCGACGACAGCCAGAAG |
| β-actin | rat-actb-F | ACGCACGATTTCCCTCTCA |
| | rat-actb-R | CTCATGCCATCCTGCGTCT |

## Results

### Effects of EA intervention on depressive behavior in CUMS rats

As shown in Figure 2, compared with the normal control group, the weight of the CUMS group decreased significantly ($P < 0.001$); EA intervention led to a significant increase in weight ($P = 0.003$). The sucrose preference rate was reduced after CUMS modeling ($P = 0.001$); this preference improved after EA intervention ($P < 0.001$). The resting time in center ($P < 0.001$) and total traveled distance ($P = 0.017$) also decreased after CUMS modeling; EA intervention reversed these behaviors ($P = 0.001$ and $P = 0.039$, respectively).

Similarly, increases in body weight ($P = 0.009$), sucrose preference rate ($P = 0.011$), resting time in center ($P < 0.001$) and total traveled distance ($P = 0.039$) were observed in the paroxetine group, compared with the CUMS group. There were no significant differences in behavioral indicators between the EA and paroxetine groups. These results suggested that EA intervention could improve depression-like behavior, in a manner similar to that of paroxetine intervention.

### Effects of EA intervention on hippocampal morphology in CUMS rats

TEM was used to observe pathological changes in depressed rats, before and after intervention. Normal morphologies of
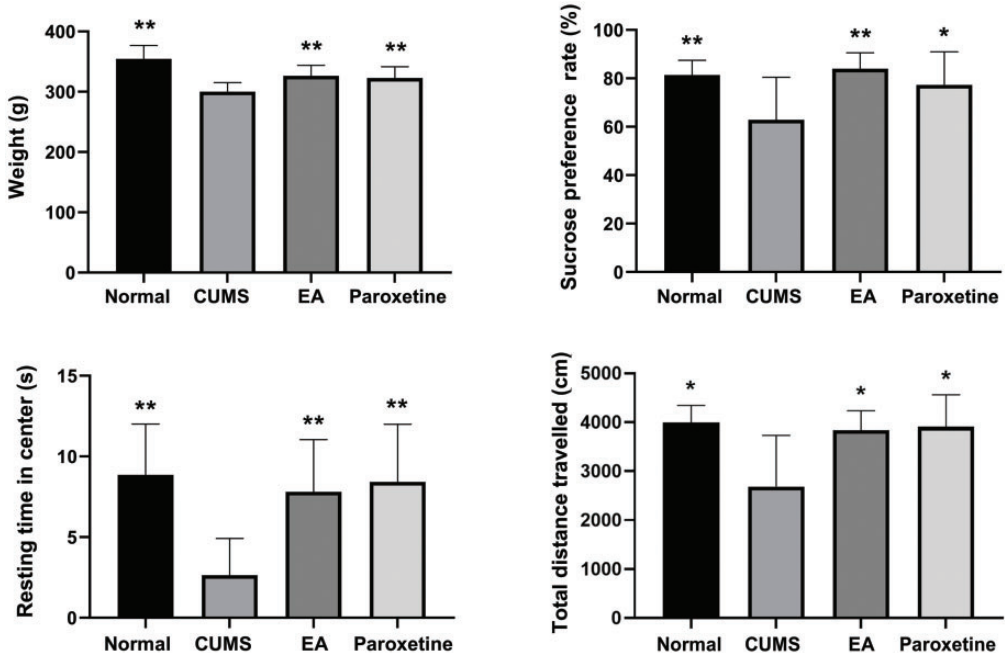


**Figure 2.** EA intervention improved depressive behavior in CUMS rats.
Behavioral test results after CUMS modeling, EA intervention, and paroxetine intervention. (a) Weight. (b) Sucrose preference rate. (c) Resting time in center. (d) Total traveled distance. Data are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$, compared with CUMS group.
Abbreviations: EA, electroacupuncture; CUMS, chronic unpredictable mild stress.

synapses, mitochondria, and Golgi apparatuses were all clearly visible in the normal control group (Figure 3a). Pathological changes in hippocampal neuronal organelles were observed after CUMS modeling (Figure 3b). In the CUMS group, the interstitial spaces of most mitochondrial cristae were swollen, the number of mitochondrial cristae had decreased, the structure was fuzzy and disordered, and mitochondrial focal cavitation was present (Figure 3b, blue arrow in model group). Although synaptic structure was discernible, the synaptic cleft was blurred, and there were few synaptic vesicles (Figure 3b, red arrow).

The severity of pathological changes in hippocampal neuronal organelles was markedly reduced after EA intervention (Figure 3c). Few mitochondria were swollen, although the number of mitochondrial cristae remained low (Figure 3c, blue arrow). The synaptic structure, presynaptic membrane, postsynaptic membrane, and synaptic vesicles were clearly visible; moreover, the postsynaptic density exhibited a long active zone (Figure 3c, red arrow). Similar reductions in pathological changes in hippocampal neurons were present after paroxetine intervention (Figure 3d).
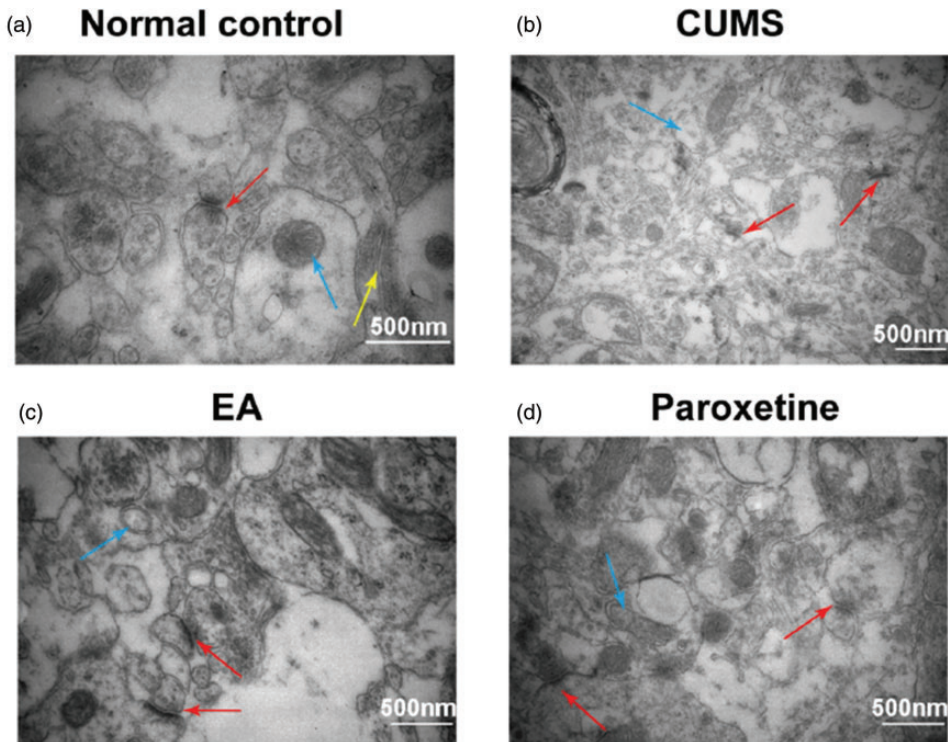


**Figure 3.** EA intervention improved the morphologies of hippocampal organelles and synapses in CUMS rats.
Transmission electron microscopy photographs of hippocampal morphology after CUMS modeling, EA intervention, and paroxetine intervention. Scale bars, 500 nm. (a) Hippocampal morphology in normal control group. (b) Hippocampal morphology in CUMS group. (c) Hippocampal morphology in EA group. (d) Hippocampal morphology in paroxetine group.
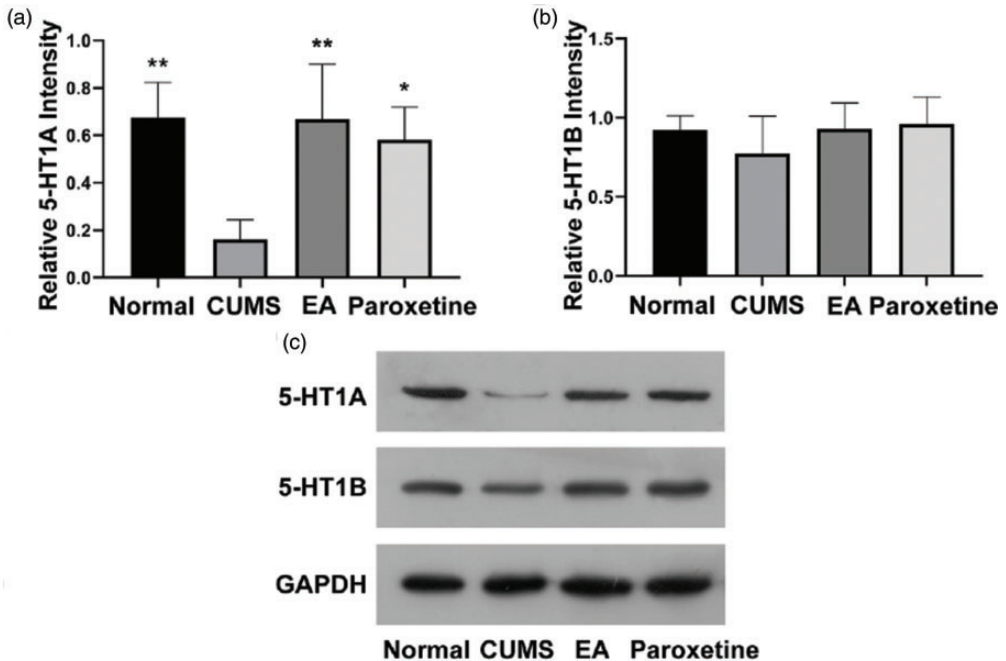Abbreviations: EA, electroacupuncture; CUMS, chronic unpredictable mild stress.

## Effects of EA intervention on protein expression levels of 5-HT receptors in the hippocampus

Western blotting revealed that the protein expression levels of 5-HT1A receptor in hippocampal tissues significantly decreased after CUMS modeling ($P = 0.004$). Notably, EA intervention resulted in upregulation of 5-HT1A receptor expression, compared with the levels observed in the CUMS group ($P = 0.005$) (Figure 4a). However, there were no obvious changes in protein expression levels of the 5-HT1B receptor after CUMS modeling and EA intervention (Figure 4b).

## Effect of EA intervention on mRNA expression levels of 5-HT receptors in the hippocampus
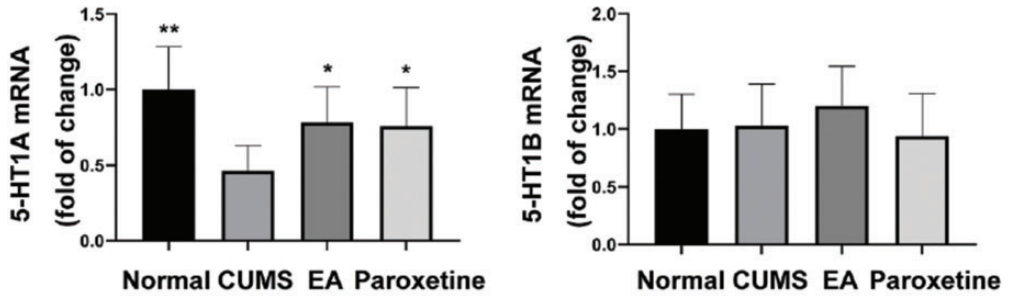
mRNA expression levels of 5-HT1A and 5-HT1B in the hippocampus on day 36 are summarized in Figure 5. mRNA expression levels of 5-HT1A receptor significantly increased after EA intervention, compared with the levels observed in the CUMS group ($P = 0.019$). Paroxetine intervention had a similar effect ($P = 0.030$). There were no significant differences in mRNA expression levels of the 5-HT1B receptor among the CUMS, EA, and paroxetine groups.



**Figure 4.** Protein expression levels of 5-HT1A and 5-HT1B receptors were altered in rat hippocampus after EA intervention.
Protein expression levels were assessed by western blotting. Results in (a) and (b) are from independent experiments performed in triplicate. (a) Relative protein expression of 5-HT1A. (b) Relative protein expression of 5-HT1B. c Representative protein bands of 5-HT1A and 5-HT1B from hippocampal tissues. In (a) and (b), data are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$ compared with CUMS group.
Abbreviations: EA, electroacupuncture; CUMS, chronic unpredictable mild stress.

**Figure 5.** mRNA expression levels of 5-HT1A and 5-HT1B receptors were altered in rat hippocampus after EA intervention.

mRNA expression levels were assessed by quantitative real-time polymerase chain reaction.

(a) Relative mRNA expression of 5-HT1A. (b) Relative mRNA expression of 5-HT1B. Data are expressed as mean $\pm$ standard deviation. $*P < 0.05$, $**P < 0.01$ compared with CUMS group

Abbreviations: EA, electroacupuncture; CUMS, chronic unpredictable mild stress.

## Discussion

In the current study, we aimed to identify the mechanism by which EA protects against depression, through evaluations of the effects of EA on hippocampal synaptic plasticity and associated 5-HT receptors. In this study, we selected paroxetine intervention as a positive control treatment. Considering the close similarities in physiological cycles between rats and humans, as well as the therapeutic delay of paroxetine and cumulative effect of acupuncture,[33,34] we selected 2 weeks after intervention was selected as the time point for assessment of therapeutic effects.

We used behavioral tests to measure changes in appetite, anhedonia, and anxiety in the experimental rats. The results of this study showed that weight, sucrose preference rate, resting time in center, and total traveled distance were all reduced after CUMS modeling, compared with the normal control group. These findings are consistent with those of recently published research,[35] which suggest that CUMS modeling successfully induces depression-like behavior in rats. Two weeks after EA intervention, the behavioral indices of CUMS rats improved significantly,

indicating that EA intervention could improve depression-like behavior.

TEM analysis revealed that CUMS modeling caused alterations in rat hippocampal neuronal organelles. Pathological changes in mitochondria and reduced numbers of synaptic vesicles influence neurotransmitter release, thereby affecting synaptic transmission. Combined with the fuzzy and disordered structure observed in the synaptic cleft, these results suggested that continuous stress stimulation may lead to changes in the morphology and structure of neurons and synapses, which affect hippocampal synaptic plasticity.[36–39] After EA intervention, improvements in hippocampal neuronal organelle lesions were observed, along with a reduced number of swollen mitochondria, an increase in postsynaptic density, and a longer active zone. Changes in hippocampal morphology observed before and after intervention were consistent with the trends of the behavioral indicators; these findings confirmed the antidepressant effects of EA, as well as the relationship of these effects with hippocampal synaptic plasticity.

The serotonin system has been confirmed to play an important role in the regulation of synaptic plasticity; the underlying

mechanism is mainly related to the activities of 5-HT receptors.[40] Previous studies have shown that the regulatory effects of 5-HT on synaptic plasticity are mainly achieved through postsynaptic 5-HT1A receptors.[41] Importantly, 5-HT1A receptors in the hippocampus are mainly distributed in the postsynaptic membrane; in the present study, we observed reductions in both the protein and mRNA expression levels of 5-HT1A receptors in the rat hippocampus after CUMS modeling, consistent with the findings of previous investigations.[42,43] We also found that EA or paroxetine treatment led to upregulation of 5-HT1A and enhanced expression of 5-HT1A receptor mRNA. These results suggest that EA achieves its antidepressant effect by enhancing the excitability of postsynaptic 5-HT1A receptors (i.e., 5-HT1A heteroreceptors).

Some studies have revealed that 5-HT1B can improve depression-like behavior by regulating synapses of lateral habenula.[44] However, in the present study, we found no significant differences in the protein and mRNA expression levels of 5-HT1B among CUMS, EA, and paroxetine groups after interventions had been completed. We presume that the discrepant results may be due to differences in 5-HT1B receptor functions among brain regions.

Overall, our findings suggested that changes in protein and mRNA expression levels of 5-HT1A receptor in the hippocampus, coupled with changes in hippocampal synaptic plasticity, might be the key pathological mechanism of depression. However, this study had some limitations. First, to ensure TEM image quality, slightly different image magnifications were used for different groups. However, we presume that the results were not affected by this change in magnification. Second, although we carefully evaluated each organelle and synaptic structure, and performed detailed mRNA expression analysis of 5-HT1A and 5-HT1B receptors in the hippocampus,

we did not analyze expression among specific regions of the hippocampus; notably, the functions of these receptors may differ among regions of the hippocampus. Third, in this study, we focused only on the mechanism by which EA protects against depression. If we had included a combination treatment group (EA and paroxetine), we might have been able to better investigate the rapid onset mechanism regarding the effects of EA combined with paroxetine. Because the levels of pro-inflammatory cytokines are significantly higher in patients with depression and anti-cytokine agents have been shown to reduce depressive symptoms (e.g., anhedonia),[45,46] further investigations are needed to determine the effects of EA intervention on pro-inflammatory cytokines.

## Conclusions

Based upon the evidence in this study regarding the regulatory effect of 5-HT on synaptic plasticity, we conclude that EA intervention can improve mRNA expression of 5-HT1A receptor, thereby promoting synaptic plasticity and alleviating depression-like behavior.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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